in body temperature and an increase in hemoconcentration invariably occurred in those which succumbed. 2. Tourniquets applied similarly to the limbs of one partner failed to produce a single death in 10 pairs of parabionts. A transient hemoconcentration was observed in the injured partner at 12 hours although rectal temperatures remained stable. Anemia occurred in the untreated member of many of the pairs, and is believed to reflect transfusion of the shocked rats, with compensatory hemodilution in an effort to sustain the blood volume.

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Inhibition of Lipid Synthesis by Alpha-Phenyl-N-Butyrate and Related Compounds. (21992)

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Cottet *et al.* have reported that feeding alpha-phenylbutyrate (phenylethylacetate) can lower serum cholesterol levels in the normal rat(1,2) and in patients with various forms of hypercholesterolemia(3). Since the drug did not prevent hypercholesterolemia in cholesterol-fed rats(1), it presumably does not act by inhibiting intestinal absorption; but the mechanism of action remains to be established. The present studies show that *in vitro* the drug strikingly inhibits synthesis of both cholesterol and fatty acids from sodium acetate-1-C^{14}.

Methods. Male Sprague-Dawley rats maintained on Purina rat chow to the time of sacrifice were decapitated and livers rapidly removed and chilled. 350-450 mg liver slices made with the Stadie-Riggs microtome were incubated with sodium acetate-1-C^{14} at 37°C in Krebs-Ringer phosphate buffer, pH 7.4, from which Ca++ and Mg++ were omitted. $C^{14}O_2$ was collected in a KOH center well and converted to $BaC^{14}O_3$ for assay of radioactivity. At the end of incubation the slices were drained of buffer and homogenized for 3 minutes in 50 ml 1:1 acetone-ethanol in a Waring blendor.* After centrifugation the supernatant was taken to dryness on a steam bath under nitrogen, and the dried residue saponified by autoclaving 4 hours at 15 pounds with 5 ml 3 N KOH. Then 5 ml absolute ethanol were added and the non-saponifiable lipid extracted with three 30-ml aliquots of n-heptane. This was back-washed with 1 N KOH and water. Aliquots of the concentrated heptane extract were dried on steel planchets and counted directly to determine ***non-saponifiable lipid radioactivity***. In several experiments

* We are indebted to Dr. Joseph H. Bragdon for this simple procedure which he has found to give essentially complete extraction of liver lipids.

cholesterol radioactivity was measured by preparing and counting the digitonide. Results were essentially the same when obtained by direct counting of heptane extracts. On the basis of this correlation, the incorporation into *non-saponifiable lipid* fraction may be taken as a measure of cholesterol synthesis. The KOH phase, after extraction of cholesterol was acidified and the alcohol and volatile fatty acids removed by heating on a steam bath. Non-volatile fatty acids were extracted with three 30-ml aliquots of n-heptane and backwashed with 10% acetic acid and water. This treatment, as shown by zero time control studies, effectively removed all traces of the original labeled acetate. After concentration, aliquots of this extract were plated and counted directly (*non-volatile fatty acid radioactivity*). Acetoacetate was decarboxylated at end of incubation by addition of aniline citrate to the acidified medium(4). The $C^{14}O_2$ liberated, representing carboxyl-carbon, was collected in a KOH center well and assayed as for metabolic $C^{14}O_2$. Inhibitors† were made to the appropriate final concentrations in buffered medium as sodium salts. All radioassays were carried out using the windowless flow proportional counter of Robinson(5). Self-absorption corrections on $BaCO_3$ and cholesterol digitonide samples were made to the 3.1 mg/cm^2 point.

Results. In vitro studies with alpha-phenyl-n-butyrate. At 1 x 10^{-3} M alpha-phenylbutyrate strongly inhibited incorporation of sodium acetate-1-C^{14} into cholesterol (*non-saponifiable lipid*). In the typical experiment summarized in Table I the rate of incorporation in presence of the drug was only about one-half that of control. Extent of inhibition did not appear to be a function of the time of incubation. However, it did vary from rat to rat. In 6 experiments with incubation times ranging from ½ to 2 hours, inhibition varied from 32% to 62%, mean 42%. The reason for this variation, as well as for variation in rate of incorporation in normal control rats noted by many laboratories, is not entirely understood(6-8).

† Alpha-phenyl-n-butyric acid was purchased from Distillation Products Industries; α,α′-diphenylbutyrate and β,β′-diphenyl-α-ethylpropionate were synthesized in Dr. Evan C. Horning's laboratory; α,α′-diphenylvalerate and 1-dimethylamino-2,2 -diphenylpentane HCl were made by Smith, Kline and French Laboratories (SKF 2314 and SKF 3019 A).

TABLE I. Effect of Alpha-phenylbutyrate on Acetate-1-C^{14} Incorporation into Lipids.*

Flask	Conc. of alpha-phenyl-butyrate	Total radioactivity in lipid fractions (10^4 c.p.m./g liver slices) Non-saponifiable lipid	Non-volatile fatty acids
1	0	1.06	.093
2	0	1.00	.088
3	1 × 10^{-3}M	.48	.030
4	"	.61	.040

* 30 min. incubation. 400-420 mg liver slices in 3.5 ml medium. 1 μc sodium acetate-1-C^{14} added (1 mc/mM).

Incorporation into non-volatile fatty acids was also inhibited to about the same extent. In 5 experiments it ranged from 23% to 62% with a mean of 41%.

The observed inhibition of both cholesterol and fatty acid synthesis from acetate suggested that the site of action of the drug might be at an early step common to both biosynthetic pathways. Incorporation of labeled acetate into acetoacetate was measured and, as shown in Table II, was also inhibited by about 50%.

At 1 x 10^{-3} M the drug did not significantly affect the endogenous oxygen consumption of liver slices (Table II), suggesting that the effects on lipid synthesis are not due to a basic disruption of the over-all tissue metabolism. This is further indicated for, in a number of experiments the oxidation of labeled acetate to $C^{14}O_2$ was decreased only by 5-15% (see Table II). Apparently the reactions of tricar-

TABLE II. Effects of Alpha-phenylbutyrate on Oxygen Uptake, Acetate-1-C^{14} Oxidation and Incorporation into Acetoacetate.*

Phenylbutyrate concentration	Total radioactivity (10^4 c.p.m./g liver slices) Acetoacetate	CO_2	Oxygen uptake (μl/g wet wt)
0	.80	2.34	927
1 × 10^{-3}M	.41	2.06	896

* 120 min. incubation. 430-480 mg liver slices in 3.0 ml medium. 0.1 μc sodium acetate-1-C^{14} added (1 mc/mM).

TABLE III. Effects of Compounds Structurally Related to Alpha-phenylbutyrate on Acetate-1-C^{14} Oxidation and Incorporation into Non-saponifiable Lipid Fraction.

Inhibitor	Total radioactivity (10^4 c.p.m./g liver slices) CO_2 Control	CO_2 Inhibited	Non-saponifiable lipid Control	Non-saponifiable lipid Inhibited
α, α′-diphenylbutyrate (1×10^{-3}M)*	21.0	20.0	1.02	.36
β, β′-diphenyl-α-ethyl propionate (1×10^{-3}M)*	21.0	16.6	1.02	.44
α, α′-diphenylvalerate (1×10^{-3}M)†	24.3	12.2	.92	.17
Idem (1×10^{-5}M)‡	14.4	10.1	.031	.025

* 120 min. incubation. 500 mg liver slices in 5 ml medium. 1 μc (= 1 μM) acetate-1-C^{14} added.

† 60 min. incubation. 380-400 mg liver slices in 3.5 ml medium. 1 μc acetate-1-C^{14} added.

‡ 60 min. incubation. 400-410 mg liver slices in 3.5 ml medium. 150 μM unlabeled acetate with 1 μc (= 1 μM) of acetate-1-C^{14}.

boxylic acid cycle and reactions of terminal oxidation can proceed with little or no impairment in the presence of these concentrations.

Inhibition of $C^{14}O_2$ production, while on the average considerably less than inhibition of lipid synthesis (mean of 7 experiments 15%), was nevertheless highly significant. In 2 experiments this inhibition rose to about 40%. This finding, as discussed further below, suggests that there is inhibition either in activation of acetate(9) or in condensation reaction through which acetyl-coenzyme A (acetyl CoA) enters the tricarboxylic acid cycle(10). When the concentration of alpha-phenylbutyrate was raised to 1 x 10^{-2} M, incorporation of labeled acetate into cholesterol and fatty acids was almost completely inhibited and incorporation into acetoacetate was inhibited by more than 70%. However, at this concentration there was also inhibition of oxygen consumption, as much as 25% in some experiments, and inhibition of the rate of incorporation of alanine-1-C^{14} into liver proteins(11). At this higher concentration, then, the drug interfered with tissue respiration and presumably also with energy metabolism.

In vivo studies with alpha-phenyl-n-butyrate. It has been proposed(3) that alpha-phenyl-butyrate might interfere with cholesterol synthesis by forming an extremely stable CoA complex, thus reducing the effective levels of the coenzyme. The concentrations of CoA in livers of rats fed alpha-phenylbutyrate, 200 mg/kg/day for 4 weeks, were measured (12). The values found for control animals were: 163, 228, 179 units/g; for the drug-fed animals, 203, 207, 226 units/g.‡ While the results in such a small group cannot be considered conclusive they suggest that any dramatic effects on levels of available CoA are not to be expected. Since the experiments of Klein and Lipmann(13) show that liver CoA must fall to 50 or 60 units/g before lipid synthesis is inhibited it is unlikely that availability of CoA is a limiting factor in lipid synthesis of phenylbutyrate-fed rats. Total liver cholesterol in these drug-fed animals also remained at normal levels.

In vitro studies with related compounds. Several compounds structurally related to alpha-phenylbutyrate have been studied *in vitro* in a similar manner. As shown in Table III, 2 of these (α,α′-diphenylbutyrate and β,β′-diphenyl-α-ethyl-propionate) have similar effects. At 1 x 10^{-3} M they inhibit cholesterol synthesis by somewhat over 50% without markedly inhibiting acetate oxidation. The third compound (SKF 2314 = α,α′-diphenylvalerate) is a considerably more potent inhibitor of cholesterol synthesis, effective even at concentrations of 1 x 10^{-5} M. These compounds, aromatic acids with structures close to that of alpha-phenylbutyrate, have been previously shown by Brodie *et al.*(14) and by Cook *et al.*(15) to inhibit a number of reactions in biotransformation of drugs. The spectrum of reactions inhibited is quite broad (*e.g.* hydroxylation, barbiturate side-chain oxidation, dealkylation) but they share in common a requirement for oxygen and triphos-

‡ We wish to thank Dr. Earl R. Stadtman for his assistance in the CoA analyses.

phopyridine nucleotide and localization in microsomes(13).

Studies by La Du *et al.*(16) have shown further that the carboxyl group is not an absolute structural requirement for inhibition in microsomal systems. 1-dimethylamino- 2,2-diphenylpentane hydrochloride (SKF 3019 A) proved extremely potent in inhibiting the dealkylation reaction. It was of interest, then, to test this compound in our system. At 1 x 10^{-3} M it inhibited acetate incorporation into cholesterol by 53% and acetate oxidation to CO_2 by 17%.

Discussion. These data suggest that the effect of alpha-phenylbutyrate on acetate incorporation into lipids represents inhibition at one of the earliest levels of acetate metabolism, probably before formation of aceto-acetate. The site of inhibition could be either: 1) in the formation of acetyl-CoA; or 2) in subsequent acetylation reactions involving acetyl-CoA; or 3) both.

The fact that incorporation into lipids was generally inhibited more strongly than oxidation to CO_2 does not rule out inhibition at the activation step. Since the reaction pathway to CO_2 is favored (Table III), the percentage inhibition in this direction, when the supply of acetyl-CoA is decreased, might well be less than the percentage inhibition in less favored pathway toward cholesterol and fatty acid synthesis.

Under 2) above are included key reactions of acetylation of oxalacetate to form citrate and acetylation of acetyl-CoA to form aceto-acetyl-CoA. A differential inhibition of these 2 acetylation reactions would account for the observation that CO_2 formation was inhibited less than incorporation into lipids. Inhibition of acetoacetate formation by ethylthioacetate and benzoate has been demonstrated by Avigan, Quastel, and Scholefield(17). They showed that these compounds probably exert their inhibitory effects as CoA derivatives. In a similar manner alpha-phenylbutyrate may inhibit only after formation of an acyl-CoA complex.

Whether *in vitro* inhibition of cholesterol synthesis from acetate described here represents the mechanism for *in vivo* effects on serum cholesterol levels reported by Cottet *et al.*(1-3) is not indicated by these studies and is the subject of further investigation.

The activity in our system of the drug potentiators studied by Brodie *et al.* is provocative. While the compounds may be acting in different ways in the 2 systems, further studies are called for to determine whether the manifold effects on drug metabolism may be mediated through effects related to the effects of alpha-phenylbutyrate on acetate metabolism.

Summary. 1) Alpha-phenyl-n-butyrate at 1 x 10^{-3} M has been shown to decrease rate of incorporation of acetate-1-C^{14} into cholesterol and into fatty acids by rat liver slices. 2) Incorporation of acetate into aceto-acetate is inhibited about the same extent, while oxidation to CO_2 is generally less strongly inhibited. 3) Endogenous oxygen consumption can proceed relatively unimpaired in the presence of 1 x 10^{-3} M alpha-phenylbutyrate. 4) Several compounds structurally related to alpha-phenylbutyrate are also demonstrated to inhibit acetate incorporation into lipids. 5) The possible relation between present observations on inhibition of lipid synthesis and studies of Brodie *et al.*(14) on drug potentiators is discussed.

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A Method for Quantitating Flexibility Changes of Pelvic Joints in Rats and Mice.* (21993)

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An increase in flexibility of the pelvic joints in mice occurs during pregnancy and following appropriate hormonal treatment(1-3). This increase was determined by manual manipulation of the pelvis. A more reliable method, suitable for both the mouse and rat, was devised to measure small differences which can be statistically analyzed.

Materials and methods. To quantitate changes in flexibility of the sacroiliac joints the animal is sacrificed, skinned, its body divided at the level of the iliac crests, and the tail severed at the 3rd caudal vertebra. The muscles, which arise in part from the dorsal aspect of the sacrum and 1st caudal vertebra to insert on the extremities, are severed at their origins and reflected laterally. The extremities and pelvic viscera are then removed. All of the musculature attached to the innominate bones is severed close to the bone, except the iliac origins of the sacrospinalis, the iliococcygei (abductor caudae internus), and the pubococcygei (abductor caudae externus; Fig. 1, PC) which are left intact. (Careless removal of the iliac origin of the sacrospinalis might damage the dorsal ligaments of the sacroiliac joint (Fig. 1, SI); the latter 2 muscles hold the ischial tuberosities in a convenient position for making subsequent measurements. Preliminary tests showed that the weight used to flex the sacroiliac joint produced maximum flexion whether or not these muscles were present.) A straight dissecting needle is inserted into the neural canal as far as it can go under moderate pressure (Fig. 1, C). The free end of the needle is slipped into an adjustable clamp which holds the specimen in a horizontal position 2 inches from a transparent plastic protractor (Fig. 1, B. J. M). The specimen's position is adjusted so that the zero line of the protractor is aligned with the dorsal-most portion of the left iliac crest and ischial tuberosity (Fig. 1, F'). To assure constant, correct alignment the positioning of the specimen is performed while viewing it through a hole (4 mm in diameter) in the center of an opaque rectangle (3 x 5 inches) placed 5 inches from the protractor (Fig. 1, A, L). A 50 g weight sufficient to produce a 3°-5° displacement of the ischial tuberosity in normal rats or a 2 g weight sufficient to produce a 1° displacement in normal mice is suspended from the symphysis pubis (Fig. 1, K). A raising of the protractor to realign the zero line with the dorsal-most portion of the iliac crest is necessary at this time because the crest is slightly elevated as the ischial tuberosity is lowered by the weight. While viewed through the hole in the rectangle a movable transparent plastic arm attached to the protractor is lowered by the observer's finger so that a black line running through the length of the arm aligns with the dorsal-most portion of the now displaced ischial tuberosity (Fig. 1, I, G'). Displace-

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